DEVELOPMENTAL BIOLOGY 168, 503–513 (1995)

Monoclonal Antibody MT2 Identifies the Urodele α1 Chain of Type XII Collagen, a Developmentally Regulated Extracellular Matrix Protein in Regenerating Newt Limbs

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Accepted December 1, 1994

We previously described the upregulation of the MT2 antigen during urodele limb regeneration and characterized the MT2 antigen as a 310- to 325-kDa chondroitin-sulfated glycoprotein with a core protein of 285–300 kDa. In this study, we screened a newt blastema cDNA library using monoclonal antibody (mAb) MT2 and obtained a 1-kb cDNA fragment, designated Isolate (IS)-1. Subsequent screening of the same library using IS-1 cDNA as a probe provided IS-2, a 2.8-kb cDNA. IS-2 overlaps IS-1 at its 5′ end, is highly homologous to a portion of the α1 chain of the chicken type XII collagen cDNA (α1[XII]), and spans a third of the chicken α1[XII] cDNA, from the last 62 amino acids of the second A domain of von Willebrand factor to the first two repeats of the fourth fibronectin type III domain. The peptide sequence deduced from cDNA IS-2 demonstrates invariable tryptophan, leucine, threonine, and tyrosine residues that are highly conserved among all the fibronectin type III domains within IS-2 and between corresponding sequences of IS-2 and chicken α1[XII]. A Northern blot showed a 10-kb band that corresponds to the size of the chicken α1[XII] mRNA. A fusion gene was constructed by inserting the IS-2 cDNA downstream from the *malE* gene of *Escherichia coli*, which encodes maltose-binding protein (MBP). The isopropyl β-D-thiogalactoside-induced fusion protein had the expected molecular weight and reacted to both mAb MT2 and rabbit anti-MBP serum. We conclude that mAb MT2 identifies the urodele α1[XII].

The expression pattern of the type XII collagen gene in newt limb regenerates was examined by *in situ* hybridization. Type XII collagen transcripts first appeared at 3 days after amputation in cells of the basal layer of the wound epithelium. At Day 10, both the basal wound epithelial cells and the distal mesenchyme cells were highly transcriptionally active. At mid-bud and late-bud blastema stages, wound epithelium expression had decreased, whereas the mesenchyme remained strongly active in transcription and showed a tendency toward distal regionalization. Condensing cartilage showed no signal. Finally, at the late digit stage, hybridization became largely restricted to the perichondrium. The *in situ* results suggest a developmental role for type XII collagen in regeneration.

INTRODUCTION

The regenerating salamander limb has been a valuable system for addressing several problem areas of limb development. Upon amputation of a newt or an axolotl limb there is a sequence of events set into motion that results in the precise replacement of the missing parts. These events include cellular dedifferentiation and histolysis in the distal stump, migration and proliferation of the dedifferentiated cells, blastema growth, redifferentiation, and pattern formation (Chalkley, 1954; Iten and Bryant, 1973).

The synthesis and degradation of extracellular matrix (ECM) molecules (e.g., collagen, laminin, fibronectin, hyaluronic acid, and tenascin) have been implicated as being important for the initiation and progression of limb regeneration (Mailman and Dresden, 1976; Gulati *et al.*, 1983; Mescher and Munaim, 1986; Onda *et al.*, 1990). One way that cells interact with the ECM is to actually bind to the cell binding domains of ECM molecules as in the binding of cells to GRGDSP sequences of fibronectin (Ruoslahti and Pierschbacher, 1987) and the RGD-containing sequences of other matrix molecules (Adams and Watt, 1993). A number of perturbation experiments, including addition or subtraction of ECM molecules and use of blocking antibodies, show the importance of both positive and negative regulation of development by ECM molecules (reviewed in Adams and Watt, 1993). For example, exogenously supplied ECM molecules regulate both production and organization of ECM by mammary

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0012-1606/95 $6.00

epithelial cells (Streuli and Bissell, 1990). Also, fibronectin added to keratinocytes in culture inhibits their differentiation (Adams and Watt, 1993), and fibronectin inhibits, while laminin enhances, endothelial cell differentiation (Kubota *et al.*, 1988).

By using monoclonal antibodies (mAbs) and DNA cloning techniques, we have characterized several ECM molecules that have potential significance in limb regeneration, including MT1 (tenascin; Onda *et al.*, 1990, 1991), ST1 (Yang *et al.*, 1992), MT2 (Klatt *et al.*, 1992), and MT4 (fibronectin; Nace and Tassava, 1994). Previous studies in our laboratory indicate that monoclonal antibody MT2 recognizes an extracellular matrix glycoprotein that is upregulated during limb regeneration of adult salamanders but is absent from developing limb buds (Klatt *et al.*, 1992; Tassava *et al.*, unpublished data). During limb regeneration, the MT2 antigen is colocalized with tenascin in intact and regenerating limbs. In addition, Western blots showed that MT2 is a 310- to 325-kDa chondroitin-sulfated glycoprotein with a core protein of 285–300 kDa (Klatt *et al.*, 1992).

Since the MT2 antigen is upregulated in regenerating but not developing limbs of the urodele amphibian, it is worthwhile to further characterize this antigen in terms of its molecular identity, gene expression pattern, and role in limb regeneration. Here we describe the isolation and molecular characterization of λgt11 clones of newt MT2 cDNAs. We show that two MT2 cDNAs obtained are highly homologous to portions of the chicken α1 chain of type XII collagen (α1[XII]) cDNA and that the MT2 mRNA is about the same size as that of the chicken α1[XII] cDNA. Moreover, we show that the expression of the MT2 gene in the regenerating limb is developmentally regulated. Together these results suggest that mAb MT2 recognizes the urodele α1 chain of type XII collagen and that this ECM protein may function during limb regeneration.

MATERIALS AND METHODS

Animals

Newts (*Notophthalmus viridescens*) were collected from ponds in southern Ohio, maintained in aerated tap water, and fed beef liver three times weekly. Forelimb amputation was performed at the mid-radius/ulna level while animals were anesthetized with neutralized 0.15% MS222 (ethyl *m*-aminobenzoate methanesulfonate; Sigma). The protruding bones were trimmed and the animals were kept on ice for 2 hr before returning to aerated water at room temperature (24 ± 1°C).

Isolation of Newt MT2 cDNA Clones

mAb MT2 was used initially to screen a newt mid-bud blastema cDNA library in λgt11 (a kind gift from J. Brockes). Duplicate filters from 10 plates with approximately 1×10^6 plaque-forming units in *Escherichia coli* strain Y1090 were screened with a 1:100 dilution of mAb MT2 in 1% nonfat dry milk. For detection of positive clones, filters were incubated in a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG and visualized with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Positive plaques that showed mAb MT2 reactivity were isolated, and the cDNA insert from one of the positive plaques, designated isolate-1 (IS-1), was released from the purified phage DNA upon *Eco*RI digestion. The insert was recovered from an agarose gel after electrophoresis, denatured by boiling, and labeled with [^{32}P]dATP using a random priming DNA labeling kit (Boerhinger Mannheim). The labeled insert was then used as a probe to hybridize another set of duplicate filters immobilized with 1×10^6 plaque-forming units from the mid-bud blastema cDNA library. The filters were prehybridized for 3 hr at 50°C with 6× SSC, 1× Denhart's, 1% SDS, and 100 μg/ml boiled herring sperm DNA (Amresco) and then hybridized with 1×10^6 cpm/ml probe at 50°C in fresh prehybridization solution overnight. The filters were washed twice with 2× SSC, 0.1% SDS at room temperature for 5 min and twice with 0.1× SSC, 0.1% SDS at 60°C for 10 min before autoradiography. The positive plaques were recovered from the agar plates, and the phage particles were purified, digested with *Eco*RI, and resolved on a 0.7% agarose gel by electrophoresis. The DNAs were transferred to a sheet of nitrocellulose filter and detected by Southern blot using the same probe and same conditions as above for the library screening. All positive inserts were subcloned into the *Eco*RI site of pBluescript SK+ (pBSSK+; Stratagene) using standard methods.

Maltose Binding Protein/IS-2 Fusion Protein Generation and Immunoblotting

The *Xba*I/*Hin*dIII fragment of pBSSK+ containing a 2.8-kb MT2 cDNA (IS-2) was cloned into the pMAL-c2 vector (New England Biolabs) downstream from the *MalE* gene of *E. coli*, which encodes maltose-binding protein (MBP). The fusion gene was transformed into *E. coli* strain DH5α and induced with 0.3 mM isopropyl β-D-thiogalactoside (IPTG) for fusion protein expression. Meanwhile, the same bacterial culture without addition of IPTG was used as a control for the basal expression of the fusion gene. To ensure the specificity of the later immunoblot analyses, the pMAL-c2 vector alone, which carries the *MalE*/β-*gal*-α gene, was also transformed into DH5α, and MBP was expressed under both inducing and noninducing conditions. All cells were harvested by

centrifugation at 4000*g* for 10 min at 4°C, and the pellets were resuspended in 10 m*M* Tris–HCl, pH 7.4, 200 m*M* NaCl, 1 m*M* EDTA, and 10 m*M* β-mercaptoethanol (10 ml/g of wet cells). The samples were frozen at −20°C overnight, thawed in cold water, and sonicated. The bacterial debris was removed by centrifugation at 9000*g* for 30 min, and the protein concentration in the supernatant was measured using the Bio-Rad protein assay. For immunoblotting, a 10-μg protein sample from each extraction was diluted 1:1 with 2× SDS sample buffer (100 m*M* Tris–HCl, pH 6.8, 200 m*M* dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiled for 10 min. The samples were electrophoresed on duplicate 12% SDS–PAGE gels and electrophoretically transferred onto nitrocellulose. The Western blot procedure was as described by Klatt *et al.* (1992) except 1:400 dilutions of the mAb MT2 or 1:10,000 dilutions of the rabbit anti-MBP serum (New England Biolabs) were used. The blots were further incubated with a 1:2000 dilution of alkaline phosphatase-conjugated goat-anti-mouse IgG (for mAb MT2) or goat-anti-rabbit IgG (for rabbit anti-MBP serum), followed by visualization with NBT and BCIP. Both alkaline phosphatase-conjugated IgGs were obtained from Organon Teknika.

DNA Sequencing

A series of plus single-stranded cDNA IS-2 subclones in pBSSK+ was generated using standard methods (Vieira and Messing, 1987) and restriction sites found in the IS-2 cDNA. The templates were sequenced using the dideoxy method with the Sequenase version 2.0 DNA sequencing kit (USB) following the manufacturer's directions. The sequence data were compiled using the DNASTAR alignment program and homologous sequences were searched using the BLAST network service at the National Center for Biotechnology Information (Altschul *et al.*, 1990).

Total Blastema RNA Extraction and Northern Blot

Total RNAs from 30 mid-bud forelimb blastemas and 15 2-week tail blastemas of newts were extracted using the TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. For Northern blotting, 15 μg of total RNA was prepared in a solution with a final concentration of 1× Mops running buffer, 2.2 *M* formaldehyde, and 50% deionized formamide. After incubation at 55°C for 15 min, 10 μl of formaldehyde loading buffer (80% formamide, 0.01 *N* NaOH, 1 m*M* EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added, and the mixture was loaded onto a 0.7% agarose gel made in 1× Mops and 2.2 *M* formaldehyde. The gel was run at 5 V/cm for 1.5 hr and depurinated with 0.05 *M* NaOH/1.5 *M* NaCl for 30 min, followed by neutralization with 0.5 *M* Tris–HCl (pH 7.4)/1.5 *M* NaCl for 20 min. The RNA species were then transferred to Nytran membrane (Schleicher & Schuell) overnight with 20× SSC and immobilized by use of a Stratagene UV-Crosslinker. To detect the MT2 mRNA, the membrane was prehybridized in hybridization solution (2× SSC, 1% SDS, 5× Denhardt's solution, and 300 μg/ml denatured herring sperm DNA) for 3 hr at 62°C and then hybridized overnight with 2×10^6 cpm/ml of random-primed ^{32}P-labeled IS-2 cDNA probe in prehybridization solution plus 10% dextran sulfate. The membrane was then washed twice (5 min each) with 2× SSC and 0.1% SDS at room temperature, twice (10 min each) with 0.2× SSC and 0.1% SDS at 42°C, and twice (10 min each) with 0.2× SSC and 0.1% SDS at 68°C. Finally the membrane was exposed to a Kodak X-OMAT film at −80°C for 3 days with an intensifying screen.

In Situ Hybridization

To generate MT2-specific riboprobes, a 235-bp *Eco*RV–*Eco*RI fragment from the 3′ end of the IS-1 cDNA was cloned into pBSSK+ and designated IS-1-1 (Fig. 1). To generate an antisense transcript, 500 μg of *Eco*RV-linearized IS-1-1 was used as a template for *in vitro* transcription using T3 RNA polymerase according to the manufacturer's protocol (Stratagene). The sense strand transcript was generated by the same procedure except that the IS-1-1 was linearized with *Eco*RI and transcribed using T7 RNA polymerase. All transcription reactions were performed in the presence of [^{35}S]UTP. The RNA probes were purified using Nuctrap push columns (Stratagene).

Regenerates at different stages with a small amount of stump tissue were sampled and frozen in OCT compound (Miles Inc.). Cryostat sections 10 μm thick were collected on 3-triethoxysilylpropylamine-treated slides. The sections were fixed in 4% paraformaldehyde containing 1× PBS, pH 7.2, for 20 min at room temperature, then washed twice in 1× PBS, 5 min each, and acetylated by immersing slides in 0.25% acetic anhydride in 0.1 *M* triethanolamine buffer, pH 8.0, for 10 min. The sections were finally dehydrated through a graded ethanol series. The hybridizations were carried out at 55°C in hybridization mix (50% formamide, 0.3 *M* NaCl, 10 m*M* Tris–HCl, 1× Denhardt's solution, 5 m*M* EDTA, 0.5 mg/ml yeast tRNA, and 10% dextran sulfate, pH 7.5) with either sense or antisense riboprobes at a concentration of 1×10^7 cpm/ml of hybridization mix for 16 hr.

Slides were washed twice in 2× SSC, for 5 min each, and then in wash solution I (50% formamide, 2× SSC, and 0.1% β-mercaptoethanol) at 55°C for 30 min. The

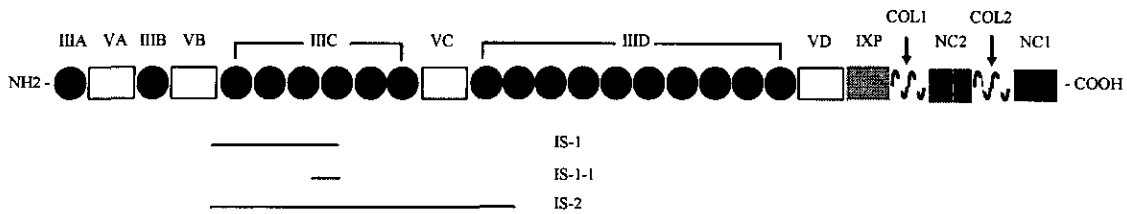

FIG. 1. Graphic representation of the alignment of cDNAs IS-1, IS-1-1, and IS-2 with the chicken α1[XII] collagen mRNA sequence (CHKCOLFIB; Yamagata *et al.*, 1991). cDNA IS-2 overlaps IS-1 and shows homology with CHKCOLFIB at both the nucleotide level (69%) and the amino acid level (65%; or 79% if all compatible amino acids are also counted) in the region spanning the last 62 amino acids of the second von Willebrand A domain (VB) to the first two repeats of the fourth fibronectin type III domain (IIID); IS-1-1 is a subclone of IS-1 cDNA and it served as a template for the synthesis of riboprobes used for *in situ* hybridization (see Materials and Methods). IIIA–IIID, repeats showing homology to the fibronectin type III motif; VA–VD, units of homology to the A domain of von Willebrand factor; IXP, the region of homology to the NC4 (noncollagenous 4) domain of type IX collagen. COL 1 and COL 2, the two collagenous regions comprising 7.7% of α1[XII]; NC1 and NC2, the noncollagenous domains 1 and 2. The domain spanning IIIA to IXP is also known as the NC3 domain. The NC2 domain of the chicken α1[XII] collagen contains an RGD sequence implicated in cell-matrix interactions (Yamagata *et al.*, 1991).

slides were then treated with RNase A (20 μg/ml) for 30 min at 37°C and further washed in wash solution I at 55°C for 30 min. Finally, the slides were washed in wash solution II (0.1× SSC and 0.1% β-mercaptoethanol) at 55°C for 30 min. The sections were then dehydrated, coated with Kodak NTB-2 emulsion (diluted 1:1 with distilled water), and exposed for 14 days at 4°C. Slides were developed with Kodak D-19 developer for 2.5 min at 15°C and fixed for 5 min with Kodak fixer. Sections were stained with hematoxylin and counterstained with eosin. The sections were mounted, coverslipped, and viewed under dark-field microscopy.

RESULTS

Isolation and Characterization of a Newt Type XII Collagen cDNA

To determine the identity of the MT2 antigen, we screened a λgt11 cDNA library from newt blastemas with mAb MT2. Of 1×10^6 plaque-forming units, 1 positive plaque, IS-1, was identified with a high immunoreactivity to mAb MT2 and containing a 1-kb insert. Upon rescreening the cDNA library using IS-1 as a probe, 9 positive plaques were identified. These 9 phage DNAs contained cDNA inserts with lengths varying from 0.8 to 2.8 kb, and all hybridized to the IS-1 cDNA probe in Southern blots (data not shown). The 2.8-kb clone, designated IS-2 (Fig. 1), was further analyzed along with IS-1 by dideoxy DNA sequencing and the result showed that the 5′ end of IS-2 was identical to the 5′ end of IS-1 (Fig. 1). After searching through the GenBank databases, a highly homologous match of IS-2 cDNA sequences to a portion of the α1 chain of the chicken type XII collagen cDNA (α1[XII]; Yamagata *et al.*, 1991) was obtained, with an overall identity of 69% at the nucleotide level and 65% at the amino acid level (Fig. 2A). The latter will be up to 79% if all of the compatible amino acid sequences are also taken into account.

The IS-2 cDNA spans a third of the chicken α1[XII] cDNA, from the last 62 amino acids of the second A domain of von Willebrand factor to the first two repeats of the fourth fibronectin type III domain (IIID; Fig. 1). Moreover, the amino acid sequence deduced from the IS-2 cDNA demonstrated invariable tryptophan, leucine, threonine, and tyrosine residues that are found to be conserved among all the fibronectin type III domains within the IS-2 peptide and between corresponding peptide sequences of IS-2 and chicken α1[XII] (Fig. 3). Among them, the tryptophan, leucine, tyrosine, and threonine residues are also seen in the type III repeats of the deduced amino acid sequence of the newt tenascin cDNA NvTN.1 (Onda *et al.*, 1991).

As we have previously shown, the MT2 antigen is a chondroitin-sulfated glycoprotein (Klatt *et al.*, 1992). It is of interest to note that the deduced amino acid sequence of IS-2 cDNA has seven seryl-glycyl sequences that might be modified with glycosaminoglycans, five in the third fibronectin type III domain (IIIC) and two in the second A domain of von Willebrand factor (VB; Fig. 3).

A 54% identity of amino acid sequence was obtained from the comparison between the chicken collagen XIV (Waelchli *et al.*, 1993) and a portion of the IS-2 molecule (corresponding to the third A domain of von Willebrand factor in chicken α1[XII] as shown in Fig. 2B. However, the overall identity of the whole IS-2 amino acid sequence to the chicken collagen XIV is only 36%.

Immunoreactivity of the MBP/IS-2 Fusion Protein to mAb MT2

To confirm that the IS-2 cDNA encodes a peptide with MT2 antigenicity, the cDNA fragment was cloned down-

A

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Chkα1[XII]  DVEIFAVGVKDAVRTELEAIASPPAETHVYTVEDFDAFQRISFELTQSVCLRIEQELAAIRKKSYVPAKNMVFSDVTSDSFKVSWSAAGS  656
IS-2        .............S......T..TA.....................IW.......KS.KV..LT.PRDLS.AE...S..R....P.AE   90

Chkα1[XII]  EEKSYLIKYKVAIGGDEFIVSVPASSTSSVLTNLLPETTYAVSVIAEYEDGDGPPLDGEETTLEVKGAPRNLRITDETTDSFIVGWTPAP  746
IS-2        DAIA..VN.T..L..E..V.....PT..T.....F.K...E.R.V...PE.ES...K........R.......V........K.......  180

Chkα1[XII]  GNVLRYRLVYRPLTGGERRQVTVSANERSTTLRNLIPDTRYEVSVIAEYQSGPGNALNGYAKTDEVRGNPRNLRVSDATTSTTMKLSWSA  836
IS-2        .......IA...VA....KE...QG...A...Y..F...K.H..GVP.......T....NGA.E..V.E.K.....EP....A.R.T.DK  270

Chkα1[XII]  APGKVQHVLYNLHTRYAGVETKELTVKGDTTSKELKGLDEATRYALTVSALYASGAGEALSGEGETLEERGSPRNLITTDITDTTVGLSW  926
IS-2        ......RY.R...S.S..GDI..V......STTV..E..PG.A.T.S.NP.......T.VT...A..Q......D..IK......I.T..  360

Chkα1[XII]  TPAPGTVNNYRIVWKSLYDDTMGEKRVPGNTVDAVLDGLEPETKYRISIYAAYSSGEGDPVEGEAFTDVSQSARTVTVDNETENTMRVSV 1016
IS-2        .A...M.RG...A.Q..F..KT..NH...D.TNT..RN.D......L.V..N.A......LS...T.EA.PDGKI.KISE...T..KATW  450

Chkα1[XII]  AALTWEGLVLARVLPNRSGGRQMFGKVNASATSIVLKRLKPRTTYDLSVVPIYDFGQGKSRKAEGTTASPFKPPRNLRTSDSTMSSFRVT 1106
IS-2        QPAPGNV.NYRV.YRP.A....IVA..PPAV..T..R..T.L....I..I.V.KE.D..T.QGS...L...NA..SIK..EP.R.T....  540

Chkα1[XII]  WEPAPGRVKGYKVTFHPTEDDRNLGELVVGPYDSTVVLEELRAGTTYKVNVFGMFDGGESNPLVGQEMTTLSDTITEPFLSRGLECRTRA 1196
IS-2        ......E.....I....EG..GY...MM..............R.S.......V..D.Q.P..I.H.T...R.APRS.IP.S..D.T.K.  630

Chkα1[XII]  EADIVLLVDGSWSIGRPNFKTVRNFISRIVEVFDIGPDKVQIGLAQYSGDPRTEWNLNAYRTKEALLDAVTNLPYKGGNTLTGMALDFIL 1286
IS-2        Q.....................I.......V.......S.R...AVS..........Q..THK..KS.M...A.........N..S..K...  720

Chkα1[XII]  KNNFKQEAGLRPRARKIGVLITDGKSQDDVVTPSRRLRDEGVELYAIGIKNADENELKQIATDPDDIHAYNVADFSFLASIGEDVTTNLC 1376
IS-2        E...RPGV.M.EK....AI.L........I.A..K.YA...I....V...........E..S...ELYM.......L.TN.VN.L.E.V.  810

Chkα1[XII]  NSVKGPGDLPPPSNLVISEVTPHSFRLRWSPPPESVDRYRVEYYPTTGGPPKQFYVSRMETTTVLKDLTPETEYIVNVFSVVEDESSEPL 1466
IS-2        .......G.N......T..P..R...VT.V..SQ..E.FK.....VA..R.QEV..RGTQ.....VG.K.....Y...Y..EGN.I....  900

Chkα1[XII]  IGREITYPLSSVRNLNVYDIGSTSMRVRW                                                              1495
IS-2        A.T.T.L.IP....M.L....T.T...K.                                                               929
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B

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Chkα1[XIV]  NASKPTPEGNLFTCKTPAIADIVILVDGSWSIGRFNFRLVRLFLENLVSAFNVGSEKTRVGLAQYSGDPRIEWHLNAYGTKDAVLDAVRN  228
IS-2        D.PRSPIPSSGLD.T.K.Q....L..........P..KI..N.ISRV.EV.DI..DRVQIAVS.......T..Q..THK..KSLM...A.  702

Chkα1[XIV]  LPYKGGNTLTGLALTYILENSFKPEAGARPGVSKIGILITDGKSQDDVIPPAKNLRDAGIELFAIGVKNADINELKEIASEPDSTHVYNV  318
IS-2        ........N..S..KF....NFR.GV.M.EKAR..A..L........IVA.S.RYA.E....Y.V.I....E........D..ELYM...  792

Chkα1[XIV]  ADFNFMNSIVEGLTRTVCSRVEEQEK                                                                  344
IS-2        ...SLLTNI.ND..EN..NS.KGPGG                                                                  818
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FIG. 2. (A) Comparison of amino acid sequence between IS-2 and chicken α1[XII] (Chkα1[XII]) shows an overall identity of 65%. (B) A 54% identity in amino acid sequence is obtained between the chicken α1 chain of collagen XIV (Chkα1[XIV]) and a small portion of IS-2 molecule, which corresponds to the third A domain of von Willebrand factor in the chicken α1[XII]. A dot in the IS-2 indicates that the corresponding amino acid is identical to that of Chkα1[XII] or Chkα1[XIV]. Nonidentical amino acids are spelled out.

stream to the *MalE* gene in the pMAL-c2 vector. When the protein extract from the pMAL-c2-transformed DH5α was analyzed on Western blots with both mAb MT2 and rabbit anti-MBP serum, an IPTG-inducible component was detected by rabbit anti-MBP serum (Fig. 4, lanes 2 and 3) but not by mAb MT2 (Fig. 4, lanes 7 and 8). This component has a molecular weight of about 50 kDa, which corresponds to the size of MBP/β-gal-α fusion. However, when the protein extract from the *MalE*/IS-2 fusion gene-transformed DH5α was resolved on SDS-PAGE and blotted, a unique band with a molecular weight of about 145 kDa was identified by both mAb MT2 and rabbit anti-MBP serum (Fig. 4, lanes 5 and 10). The basal level of expression of this protein species in the absence of IPTG (Fig. 4, lanes 4 and 9) was much lower than that of MBP/β-gal-α fusion protein (Fig. 4, lane 2). To eliminate the possibility that mAb MT2 and rabbit anti-MBP serum reacted with bacterial proteins that were not expressed by the transfected plasmids, the same amount of proteins from the DH5α lysates was ap-

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VB      DVEIFAVGVKDAVRSELEAIATPPTATHVYTVEDFDAFQRISFELTQSIWLRIEQELKSIKV

IIIC-1  KSLTPPRDLSFAEVTSSSFRVSWSPAAEDAIAYLVNYTVALGG   EEFVVSVPAPTTSTVLTNLFPKTTYEVRVVAEYPEGESPPLKGEETTLE
IIIC-2  VRGAPRNLRVT DETTDSFKVGWTPAPGNVLRYRIAYRPVAGG   ERKEVTVQGNERATTLYNLFPDTKYHVSGVPEYQSGPGTALNGNGATEE
IIIC-3  VVGEPKNLRVSEPTTSTAMRLTWDKAPGKVQRYLRNLHSRSAG  GDIKEVTVKGDTSTTVLKELDPGTAYTLSVNPLYASGAGTAVTGEGATLQ
IIIC-4  ERGSPRDLIIK DITDTTIGTSWTAAPGMVRGYRIAWQSLFDD   KTGENHVPGDTTNTVLRNLDPETKYRLSVYANYASGEGDPLSGEATTEASPD
IIIC-5  GKIVKISEETE      TTMKATWQPAPGNVLNYRVVYRPRAGG  RQIVAKVPPAVTSTVLRRLTPLTTYDISVIPVYKEGDGKTRQGSGTTLS
IIIC-6  PFNAPRSIKTSEPTRSTFR VTWEPAPGEVKGYKITFHPEGDD  GYLGEMMVGPYDSTVVLEELRARTSYKVNVFGVFDDGQSPPLIGHETTTLR

        DAPRSPIPSSGLDCTTKAQADIVLLVDGSWSIGRPNFKIVRNFISRVVEVFDIGSDRVQIAVSQYSGDPR
VC      TEWQLNTHKTKKSLMDAVANLPYKGGNTNTGSALKFILENNFRPGVGMREKARKIAILLTDGKSQDDIVA
        PSKRYADEGIELYAVGIKNADENELKEIASDPDELYMYNVADFSLLTNIVNDLTENVCNSVKGPG

IIID-1  GLNPPSN LVTSEPTPRSFRVTWVPPSQSVERFKVEYYPVAGG   RPQEVYVRGTQTTTVLVGLKPETEYYVNVYSVEGNEISEPLAGTETTL
IIID-2  PIPSVRNMNLYDIGTTTMR VKW
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Consensus sequence in chicken α1[XII] collagen fibronectin type III domain C:

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----------W----------L--L---T-Y----------T---
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FIG. 3. The amino acid sequence deduced from cDNA IS-2. The sequences homologous to the third and fourth fibronectin type III domains (IIIC and IIID) in the chicken α1[XII] collagen (Yamagata *et al.*, 1991) are aligned into corresponding repeats showing the invariable tryptophan, leucine, threonine, tyrosine, and glycine residues (bold). The potential residues for attachment by chondroitin sulfate are underlined in the sequence. All the invariable residues in the IS-2 amino acid sequence, except the glycine, are conserved as well among the corresponding fibronectin type III repeats in the chicken α1[XII] collagen. A similar pattern is also exhibited by the fibronectin type III repeats deduced from the newt tenascin cDNA NvTN.1 (Onda *et al.*, 1991).

plied in the immunoblotting analysis. No reactivity to either of the antibodies was detected (Fig. 4, lanes 1 and 6), indicating that the protein components showing positive reactivities to mAb MT2 and rabbit anti-MBP serum were from the ectopic expression of the transfected genes.

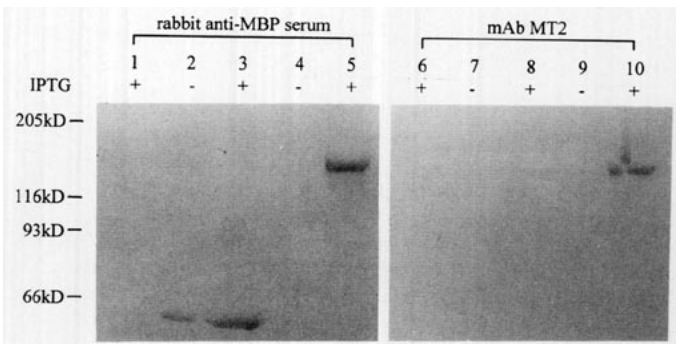


FIG. 4. Immunoblot analysis of the MBP/IS-2 fusion protein. Bacterial cultures in the absence (−) or presence (+) of IPTG were obtained from the *E. coli* strain DH5α that was either not transfected (lanes 1 and 6), transfected with pMAL-c2 (lanes 2, 3, 7, and 8), or pMAL-c2/IS-2 (lanes 4, 5, 9, and 10). The protein extracts were separated by 12% SDS-PAGE under reducing conditions. After electrophoretically transferring to nitrocellulose, the blots were reacted with either rabbit anti-MBP serum (lanes 1–5) or mAb MT2 (lanes 6–10). An anti-MBP serum reactive band of about 50 kDa in lanes 2 and 3 represents the IPTG-inducible MBP/β-gal-α fusion protein coded by the pMAL-c2 plasmid. A band of about 145 kDa, which is reactive to both rabbit anti-MBP serum (lane 5) and mAb MT2 (lane 10), represents the MBP/IS-2 fusion protein coded by the pMAL-c2/IS-2 recombinant plasmid. The molecular weight standards are indicated to the left.

Northern Blot Analysis of MT2 mRNA

IS-2 cDNA of the MT2 antigen is only a third of the size of the chicken α1[XII] cDNA. If mAb MT2 identifies a urodele counterpart of the chicken α1[XII], then the MT2 mRNA should have a size close to the chicken α1[XII] mRNA, i.e., 10 kb. To test this, Northern blot analysis was carried out on total RNA from limb and tail blastemas of the newt, using IS-2 cDNA as a probe. A single band of about 10 kb was observed in both forelimb and tail blastemal RNAs (Fig. 5).

MT2 Gene Expression in Blastemas during Limb Regeneration

Our previous studies showed that the MT2 antigen is upregulated during limb regeneration and the protein is colocalized with tenascin in intact and regenerating limbs (Klatt *et al.*, 1992). To further characterize the source of the MT2 antigen and regulation of MT2 gene expression, *in situ* hybridizations were carried out on cryosections obtained from different stages of newt forelimb regenerates. Both sense and antisense riboprobes were generated from clone IS-1-1, a 235-bp *Eco*RV–*Eco*RI fragment on the 3′ end of the IS-1 cDNA (Fig. 1). Based upon a GenBank search, this fragment

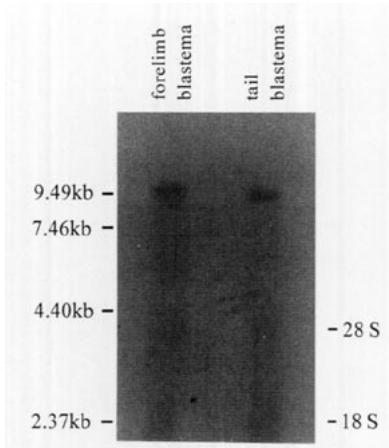


FIG. 5. Northern blot of total RNA from forelimb and tail blastemas. Fifteen micrograms of total RNA from each source was separated by a 0.7% agarose gel and blotted to Nytran nylon membrane. The blot was hybridized with the IS-2 cDNA probe and autoradiographed. A band of 10 kb, which corresponds to the size of the chicken α1[XII] collagen cDNA (Yamagata *et al.*, 1991), was observed in both forelimb and tail blastemal RNAs. The molecular weight standards are indicated to the left. The positions of 28S and 18S ribosomal RNA are to the right.

shows a high percentage of identity to the chicken α1[XII] and corresponds to part of the third fibronectin type III domain (Fig. 1).

No riboprobe signal above background could be observed in 1- or 2-day regenerates. A significant amount of signal was first detected in the wound epithelium 3 days after amputation (Fig. 6A). At Day 7, the mesenchyme cells in the distal stump tissue began to show transcriptional activity, whereas the signal was particularly strong in the basal layer of the wound epithelium (Fig. 6B). At Day 10 after amputation, riboprobe signal was strong throughout the distal mesenchyme as well as over the basal wound epithelium (Fig. 6C). The hybridizations were specific to the MT2 transcript since the sense probe did not exhibit any significant signal on adjacent sections (Fig. 6D).

The patterns of MT2 expression at the mid-bud and late-bud blastema stages were different from those at the preblastema stages. The transcript level in the basal wound epithelium was significantly lower. Although the entire mesenchyme was active in MT2 expression, hybridization signals were stronger distally. Moreover, the mesenchyme expression of the MT2 mRNA was specifically associated with the cells which had not yet differentiated since no transcript was detected within the condensing cartilage (Fig. 6E). As differentiation continued into the final digit stages, hybridization to the MT2 transcript became largely restricted to the perichondrium (Fig. 6F).

DISCUSSION

mAb MT2 Identifies the Urodele α1 Chain of Type XII Collagen

Our previous studies showed that mAb MT2 identifies an extracellular matrix protein that is colocalized with tenascin during adult newt limb regeneration. Biochemical analysis indicated that the MT2 antigen is a chondroitin sulfate-like glycoprotein (Klatt *et al.*, 1992). The combined data of the present study strongly indicate that mAb MT2 identifies the urodele α1 chain of type XII collagen.

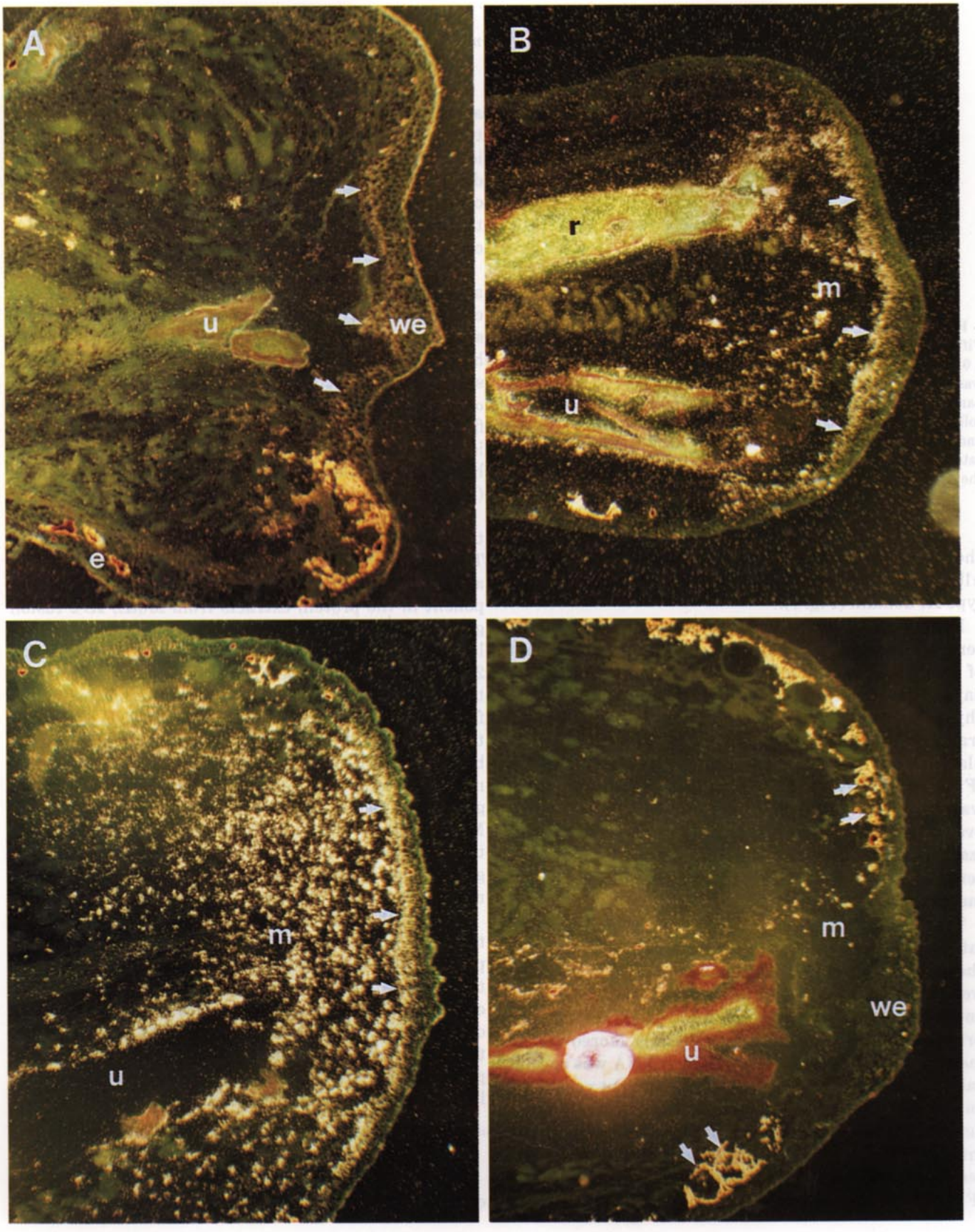
First, both the IS-2 cDNA sequence and the deduced amino acid sequence closely match a region of the chicken α1[XII] (Yamagata *et al.*, 1991), and the peptide sequence deduced from cDNA IS-2 demonstrates the highly conserved amino acid residues that are also conserved within the corresponding sequence in the chicken α1[XII] (Figs. 2 and 3). In comparison with the above findings, the IS-2 molecule showed considerably less identity to the chicken type XIV collagen, the next most homologous molecule obtained in the GenBank search (Fig. 2).

Moreover, the IS-2 cDNA codes for a peptide sequence that contains an antigenic epitope for mAb MT2 (Fig. 4). The immunoblot results clearly show that both rabbit anti-MBP serum and mAb MT2 react strongly to a component in the protein extracts from *MalE/IS-2* fusion gene-transfected *E. coli.* This component has a molecular weight of about 145 kDa and represents the MBP/IS-2 fusion protein because: (1) it does not exist in the protein extract from *E. coli* that are not transfected by the fusion; (2) it is inducible by addition of IPTG to the culture medium; (3) it contains the antigenic epitopes for both rabbit anti-MBP serum and mAb MT2; and (4) its molecular weight equals that deduced from the fusion gene (102 kDa for IS-2 and 43 kDa for MBP). The mAb MT2 reactivity of this 145-kDa component is specific to the IS-2 portion of the fusion protein since the MBP/β-gal-α fusion protein coded by the pMAL-c2 vector alone reacts only to the rabbit anti-MBP serum.

Finally, the MT2 mRNA has a molecular weight close to that of the chicken α1[XII]. Yamagata *et al.* (1991) reported that Northern blots of poly(A)+ RNA from cultured chick fibroblasts probed with a chicken type XII collagen cDNA clone gave unseparated, smeared bands of about 10 kb, consistent with the size expected from the length of the whole cDNA. The results from the present study also showed a single band of about 10 kb in both forelimb and tail blastemal RNAs (Fig. 5).

α1[XII] Gene Is Spatially and Temporally Regulated during Limb Regeneration

Although *in situ* hybridization led to a general profile of type XII collagen distribution somewhat predicted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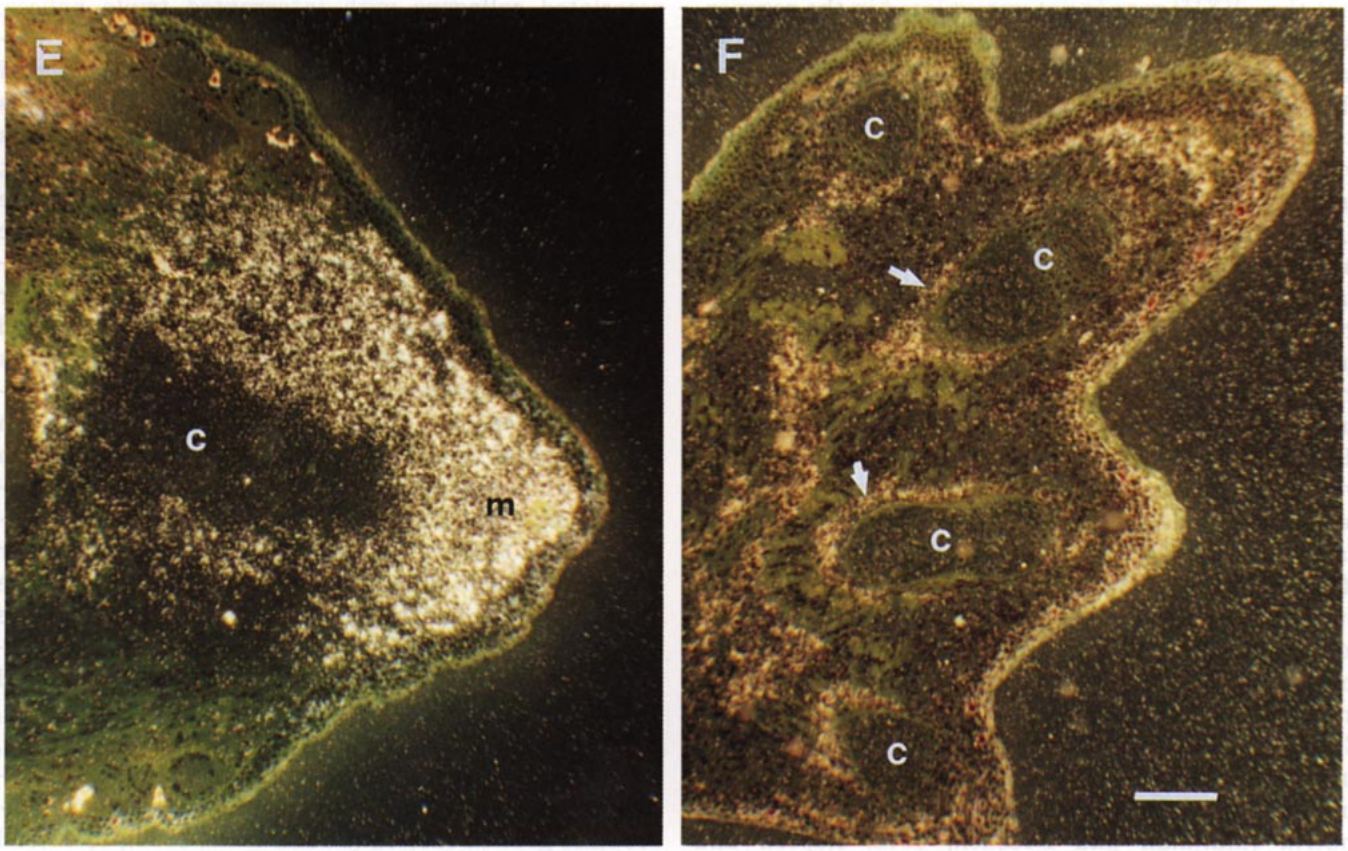

FIG. 6—*Continued*

from our previous immunostaining findings, *in situ* results are more specific in terms of the temporal and spatial regulation of gene expression. We found that the α1[XII] message was principally transcribed by the basal cells of the wound epithelium during the preblastema stage. At the mid-bud and late-bud blastema stages, the wound epithelium expression of α1[XII] message was significantly lowered, whereas the majority of blastema cells had become strongly transcriptionally active (Fig. 6). Such a transition from a dual to a single source of transcription, i.e., from both wound epithelium and mesenchyme early to mesenchyme alone as regeneration proceeds, is not unique to the α1[XII] gene. The genes of other regeneration-related ECM proteins identified in our laboratory, including fibronectin (MT4; Nace and Tassava, 1995) and tenascin (MT1; Onda *et al.*, 1991), also demonstrate similar temporal and spatial regulations. However, fibronectin and tenascin genes were turned on in the wound epithelium at 24 and 48 hr, respectively, after amputation, whereas here we show

FIG. 6. *In situ* results showing the localization of MT2 mRNA in various stages of newt forelimb regeneration. Antisense ^{35}S-labeled riboprobe transcribed from cDNA clone IS-1-1 was hybridized to cryosections of different stages of regenerates (A, B, C, E, and F) and the localization of MT2 mRNA was visualized by dark-field microscopy. (A) A micrograph of a longitudinal section of a newt forelimb 3 days after amputation. Riboprobe signal (arrows) is seen over the basal cells of the wound epithelium (we) but not in the epidermis (e) or other tissues of the stump. u, ulna. (B) A micrograph of a 7-day regenerate. Hybridization signal is strong in the basal cells of the wound epithelium (arrows). Signal is also present among the dedifferentiating mesenchyme cells (m). r, radius; u, ulna. (C) A micrograph from a 10-day regenerate. MT2 transcripts in the cells of the basal layer of the wound epithelium are still abundant (arrows). The distal mesenchyme cells (m) are strongly active in transcription. u, ulna. (D) A micrograph from a 10-day regenerate hybridized with the sense riboprobe. No nonspecific signal is seen. Pigment is associated with the skin (arrows). u, ulna; m, mesenchyme; we, wound epithelium. (E) A micrograph from a late-bud blastema. The wound epithelium signal is now very low or absent. The condensing cartilage (c) exhibits no riboprobe signal. The distal mesenchyme (m) exhibits a strong riboprobe signal. (F) A micrograph from a late digit stage regenerate illustrating a restriction of the MT2 message to the perichondrium (arrows). c, condensing cartilage. Bar, 0.1 mm.

that the α1[XII] gene is not upregulated in the regenerate until Day 3 after amputation. The unique expression patterns of these ECM genes and their sequential upregulations in regenerates suggest functions for the respective proteins in modulating the extracellular matrix toward an environment appropriate for the regeneration process to be initiated and maintained.

The importance of the wound epithelium for amphibian limb regeneration is well recognized (Singer and Salpeter, 1961). The postulated roles of the wound epithelium during limb regeneration include stimulation of mesodermal cell dedifferentiation, accumulation of these dedifferentiated cells to form a blastema, and maintenance of blastema cells in the cell cycle (Mescher, 1976; Wallace, 1981). Our previous studies show that the wound epithelium of the adult newt upregulates antigens that are identified by mAbs WE3 and WE6 (Goldhamer *et al.*, 1989; Estrada *et al.*, 1992). The expression of these antigens is largely limited to the wound epithelium throughout the regeneration process. The present results, together with the *in situ* hybridization results for fibronectin and tenascin, further emphasize the transcriptional complexity of the epithelium, as these ECM molecules are initially produced by the epithelium and then by the mesenchyme. Since dedifferentiation and cell migration require a remodeled extracellular matrix (Mailman and Dresden, 1976; Repesh and Furcht, 1982; Gulati *et al.*, 1983; Toole and Gross, 1971; Mescher and Munaim, 1986; Onda *et al.*, 1990; Arsanto *et al.*, 1990; Yang *et al.*, 1992), it is possible that the early transcription of ECM genes by the wound epithelium might provide an initial source of special ECM proteins for these processes to occur. Once the dedifferentiated cells accumulate and begin to produce their own ECM proteins, the epithelium supply of these molecules is no longer needed, and thus transcription of the α1[XII] gene, for example, ceases. Singer and Salpeter (1961) found that the basal cells of the wound epithelium of regenerating adult newt limbs exhibited ultrastructural evidence of secretory activity by virtue of their abundant rough endoplasmic reticulum. It is likely that tenascin, type XII collagen, and fibronectin represent some of the proteins secreted by the wound epithelium.

Another interesting phenomenon observed in the present study is that, although the wound epithelium has significantly lowered its transcription of the α1[XII] gene at the mid-bud and late-bud blastema stages, blastema cells that are most active in transcribing the α1[XII] gene are those directly under the wound epithelium. Thus mesenchyme cell transcription may be in response to a wound epithelium-derived signal, perhaps a growth factor (Niswander *et al.*, 1993).

Type XII collagen is a member of the FACIT (fibril-associated collagens with interrupted triple helices; Shaw and Olsen, 1991) family of collagens. These collagens are of interest because of their short interrupted collagenous domains; their noncollagenous sequences have been implicated in possible interactions with cells and with other ECM molecules (Shaw and Olsen, 1991; Yamagata *et al.*, 1991). Cell–matrix interactions have long been postulated to be important during embryonic development. Likewise, from recent studies, such interactions can be deduced to be integral to wound repair and epimorphic regeneration (see Onda *et al.*, 1990, and references therein). Our immunohistochemical studies show that, in contrast to its abundance in the undifferentiated regenerate, the MT2 antigen is not present in limb buds of urodele embryos until differentiation stages, when it exhibits the normal distribution of mature limbs (Tassava and Yang, in preparation). However, at this time we can only speculate about a role for type XII collagen in regeneration. The function of type XII collagen in regeneration might be one or more of the following: it could serve as a substratum for cell adhesion via its RGD sequence (Yamagata *et al.*, 1991) or bind to other matrix molecules such as tenascin (Klatt *et al.*, 1992), proteoglycans, or other collagens (Ninomiya *et al.*, 1990), thereby influencing growth, patterning, or differentiation. It could sequester growth factors, such as fibroblast growth factors (Poulin *et al.*, 1993), to extend their availability to cells of the regenerate or it could tie up growth factors that might otherwise inhibit growth or differentiation, therefore keeping cells dedifferentiated and proliferating. For example, the ECM molecule decorin negatively regulates TGFβ (Yamaguchi *et al.*, 1990) and SPARC (a secreted ECM protein) negatively regulates the mitogenicity of PDGF (Raines *et al.*, 1992). Moreover, type XII collagen might itself be mitogenic, as is a portion of the carboxy terminal of the long arm of laminin for PC12 cells (Kubota *et al.*, 1988).

We thank J. Brockes for providing the newt blastema cDNA library; Bjorn R. Olsen for helpful suggestions and an anti-type XII collagen antibody that was utilized in preliminary experiments; Matthew L. Poulin, Rebecca J. Gast, and MaryAnn Martin for sharing with us their technical and analytical expertise; and Tonya Y. Brady, Maera S. Flynn, Nikolas A. Jones, Hiroaki Onda, Jackie D. Nace, and Peir-en Yeh for technical assistance. We also thank Autumn Astley and Jackie Nace for their comments on the manuscript. Supported by Office of Naval Research Grant N00014-90-J to R.A.T. and a Denison University Research Foundation grant to K.P.K. E.V.Y. was supported in part by NIH Neuroscience Training Fellowship NS07291.

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